

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/00, 31/70, 39/39, 9/00 // (A61K 39/00, 31:70)		A1	(11) International Publication Number: WO 99/30733 (43) International Publication Date: 24 June 1999 (24.06.99)
(21) International Application Number: PCT/EP98/08152		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 11 December 1998 (11.12.98)		(70) Priority Data: 9726555.7 16 December 1997 (16.12.97) GB	
(71) Applicant (<i>for all designated States except US</i>): SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors; and			
(73) Inventors/Applicants (<i>for US only</i>): DALEMANS, Wilfried, L., J. [BE/BE]; (BE). VAN MECHELEN, Marcelle, Paulette [BE/BE]; (BE). BRUCK, Claudine [BE/BE]; (BE). FRIEDE, Martin [GB/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE).			
(74) Agent: DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).			
(54) Title: METHOD TO ENHANCE AN IMMUNE RESPONSE OF NUCLEIC ACID VACCINATION			
(57) Abstract			
<p>This invention provides a method to enhance an immune response of nucleic acid vaccination by simultaneous administration of a polynucleotide and a polypeptide of interest.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHOD TO ENHANCE AN IMMUNE RESPONSE OF NUCLEIC ACID VACCINATION

Field of the Invention

5 This invention relates to a method to enhance an immune response of nucleic acid vaccination by simultaneous administration of a polynucleotide and a polypeptide of interest.

Background of the Invention

10 In 1990 Wolff and colleagues (Wolff et al., *Science* 247:1465-1468 (1990)) reported that nonreplicating plasmid DNA encoding reporter genes could be internalized by muscle cells, without the use of any transfection vehicle, and that the encoded proteins were expressed following injection of the plasmid DNA. The subsequent finding that the expressed protein of "naked" (i.e., devoid of agents 15 which promote transfection) plasmid DNA was immunogenic upon intramuscular inoculation has opened a new area of research with significant clinical impact.

As such, vaccination with nucleic acids (NAVAC) has become a relatively new approach for vaccinating against a multitude of diseases. It involves the *in vivo* production of a polypeptide within a host cell rather than the more conventional 20 method of administering a polypeptide (or an attenuated, or killed microorganism) that was first produced (or cultured) *in vitro*. NAVAC also differs from more conventional vaccines in that the compound administered consists of nucleic acids, i.e., DNA or RNA, encoding selected antigen(s). Upon injection of these nucleic acids, they are taken up by the recipient cells of a mammalian host and the antigen 25 encoded by such nucleic acid(s) is subsequently expressed. Thereafter, the presence of a foreign antigen within a host can elicit a specific immune response directed against the antigen. It has been shown that NAVAC induces humoral, i.e., antibodies, as well as cell mediated immunity, i.e., T helper cell responses and 30 cytotoxic lymphocytes (see, e.g., Corr et al., *J. Exp. Med.* 184:1555-1560 (1996); Doe et al., *Proc. Natl. Acad. Sci.* 93:8578-8583 (1996)).

The induced immune responses have been shown to protect against a subsequent challenge with the infectious organism from which the antigen was originally obtained in a certain number of animal models (e.g. Ulmer et al., *Science*

59:1745-1749 (1993); Sedegah et al., *Proc Natl Acad Sci USA* 91:9866-9870 (1994); Manickan et al., *J. Immunol.* 155:259-265 (1995)). NAVAC has been shown to be much less efficient in larger animal species (when compared to administration of adjuvanted proteins), and large amounts of DNA have to be 5 administered to achieve immunisation levels comparable to that found in smaller animal species, e.g., mice (Gramzinski et al., *Vaccine Research* 5:173-183 (1996); Lu et al., *J. Virol.* 70:3978-3991 (1996); Shiver et al.; *Journal of Pharmaceutical Sciences* 85:1317-1324 (1996)). There is thus a need to improve the efficacy of 10 NAVAC, for example, by developing new delivery and targeting mechanisms, new adjuvination techniques, other routes of administration, etc. It is the object of this 15 invention to provide another improvement to the efficacy of DNA vaccination.

Summary of the Invention

In one aspect, the present invention provides a method for enhancing the 15 immune response of a nucleic acid vaccination by administration of (i) a polynucleotide encoding a gene of interest, which encodes a polypeptide and (ii) by also administering the polypeptide simultaneously, that is, during the same ongoing immune response. Preferably the administration of polynucleotide and polypeptide occur within 0-10 days of each other. One preferred embodiment is administration 20 of polypeptide 3-7 days prior to administration of polynucleotide. The polypeptide may be further prepared prior to administration such that it is presented to the immune system in such a way to provide a delayed release (e.g., encapsulated). In such a case, the polypeptide is preferably administered at the same time (concurrently) as the polynucleotide.

25 In another aspect, the present invention provides a method for enhancing the immune response of a polypeptide vaccination by administration of a nucleotide encoding a gene of interest, which encodes a polypeptide of interest, and by also administering the polypeptide simultaneously, that is, during the same ongoing immune response.

30 In further related aspects, the present invention relates to pharmaceutical compositions, vaccines, and methods to prepare such compositions and vaccines, comprising a polynucleotide encoding a gene of interest, and the corresponding

polypeptide of interest, where the ratio of polynucleotide to polypeptide is from 1000:1 to 1:1 (w/w). Optionally, the polypeptide is prepared prior to administration such that it is presented to the immune system in such a way to provide a delayed release.

5

Brief Description of the Drawings

Figure 1 (*Ig 14 post 2*) shows the serological responses in the immunised animals 2 weeks after the booster injection. The combination of the two vaccine substrates strongly enhanced the amount of RSV-FG specific antibodies compared to those obtained by using the respective substrates separately ((DNA-F or FG protein) 2x).

Figure 2 (*IgG isotype 14 post 2*) shows the isotype distribution in the different vaccine groups. All the groups who did receive a DNA vaccine at the first injection have a strong content of IgG2a, indicative for a T helper response of type 1 (Th1), whereas the protein groups have a complete bias for the opposite, that is the IgG1 isotype typical for a Th2 type response.

Figure 3 (*Lymphoproliferation*) shows the lymphoproliferation of splenocytes from the different vaccine groups upon *in vitro* restimulation with the FG antigen.

Figure 4 shows the presence of CTL's in the different vaccine groups.

Figure 5 shows the total anti-gp120 IgG titers 14 days after the second injection.

IgG titers are strongly increased in the group which received the two antigen forms (polynucleotide and polypeptide). The polypeptide (protein) administration was delayed for four days.

Figure 6 shows the isotype distribution of anti-gp120 IgG titers. Delayed protein delivery maintains considerable IgG2a titers as observed for the DNA group, which is in contrast to the protein-only group.

Detailed Description of the Invention of the Invention

The present invention relates to a method for enhancing the immune response of a mammal (e.g., mice, rabbits, primates, man) to DNA vaccines by admixing two different compounds prior to simultaneous administration. The first compound comprises a *polynucleotide* (nucleic acid) such as DNA or RNA which encodes a selected polypeptide that can stimulate protective immunity. The second compound comprises a *polypeptide*, which preferably is the same polypeptide (or

substantially the same, i.e., having the same immunodominant epitope(s)) encoded by the nucleic acid of this invention. The enhancement relates to the total increase in immune response, as determined by both humoral and cell mediated immune responses. That is, there are measurable increases in one or more of the following:

5 total antibody titer (to the selected polypeptide); in lymphoproliferation; and in CTL (cytotoxic T lymphocyte) levels. Moreover, when nucleic acid such as DNA encoding the gene of interest is admixed with the corresponding polypeptide and (simultaneously) administered to a mammal, a synergistic effect is observed. That is, not only is the DNA vaccine capable of inducing an immune response in the

10 presence of protein (polypeptide), but the presence of such protein (polypeptide) has been found to actually enhance the efficacy of the DNA vaccine. Thus, one aspect of the present invention is a composition comprising a polynucleotide and polypeptide for enhancing an immune response. Optionally, the polypeptide may be adjuvanted as well.

15 Conversely, the presence of specific nucleic acid (e.g., DNA encoding the whole or substantial parts of the polypeptide) to a polypeptide vaccine was found to increase that immune response as well. That is, the interaction of a DNA/polypeptide mixture enhances the efficacy of either individual component. This enhancement is specific to the polynucleotide added, as a similar

20 polynucleotide not encoding the specific polypeptide was unable to enhance the immune responses. One surprising aspect of the instant invention was the fact that the DNA component and the polypeptide component did not compete with each other in eliciting an immune response, and that, to the contrary, a synergistic effect was observed when DNA and protein were administered simultaneously.

25 The present invention differs from the "prime boost" approach (Barnett et al., *Vaccine* 15:869-873 (1997)) where two distinct vaccine preparations (one DNA, one protein) are prepared and administered separately, at different times, and in a specific order. That is, under the prime boost approach, a DNA molecule is administered to "prime" the immune system and at some subsequent time (weeks or

30 months later) after the primary immune response is established a protein is administered to boost the then pre-existing immune response.

Another benefit of the present invention is that in certain cases, where there is a predisposition to a strong immune response (e.g., high percentage of responders to initial vaccination, high level of antibody titers, CTL response, etc.), the combination of both compounds (DNA + protein) can obviate the need for 5 immunostimulants.

In a further embodiment of this invention are included formulations wherein the nucleic acid and protein antigen are co-administered (preferably simultaneously), but the protein antigen is encapsulated within a delayed-release vehicle which liberates the protein antigen several days after administration. Such 10 formulations enhance the magnitude of the induced immune response compared to nucleic acid alone, without biasing the immune response.

Nucleic acid

The polynucleotide materials used according to the present invention comprise 15 DNA or RNA sequences coding for polypeptides that have useful therapeutic application, e.g., prophylactic or therapeutic vaccines. According to the methods of the invention, both expressible DNA and RNA can be delivered to cells to form therein a polypeptide translation product. If the nucleic acids contain the proper control sequences, they will direct the synthesis of relatively large amounts of the 20 encoded protein. Preferably they encode antigens for organisms associated with infectious diseases caused by viruses (including but not limited to Hepatitis (all forms), HSV, HIV, CMV, EBV, RSV, VZV, HPV, polio, influenza), parasites (e.g., from the genus *Plasmodium*), and pathogenic bacteria (including but not limited to *M. tuberculosis*, *M. leprae*, *Chlamydia*, *Shigella*, *B. burgdorferi*, enterotoxigenic *E. coli*, 25 *S. typhosa*, *H. pylori*, *V. cholerae*, *B. pertussis*, etc.). Other antigens, such as found on cancerous cells (examples of known targets for immune responses to tumors are e.g., E7 for HPV-induced cervical carcinoma, Her-2/neu for breast and other cancers, 30 MAGE-3 for melanoma and other cancers, tyrosinase, melanA/MART, or gp100, a melanocyte-specific differentiation antigen) are also contemplated within the scope of this invention.

The polynucleotides of the present invention may be in the form of linear fragments, or non-linear fragments integrated into plasmids or viral DNA. The

polynucleotides (or nucleic acids) may be associated with other materials which promote transfection such as liposomal formulations, charged lipids (cationic or anionic: e.g., Lipofectin™, Transfectam™, DOTAP™, cochleates), polycations (e.g., polylysine, polyethylenimine, etc.), viral proteins (e.g., histones), peptides that bind

5 to nucleic acids (e.g., condensing agents), dendrimers, PLPG microparticles, etc.

Where the nucleic acid is DNA, promoters suitable for use in various mammalian systems are well known. For example, suitable promoters include CMV IE, RSV LTR, SV40, Adeno MLP, metallothionein, etc.

10 The exact cellular and immunological mechanisms underlying the induction of the immune response by NAVAC are not yet completely understood. A hallmark of NAVAC is the induction of cytotoxic responses, which is a part of the immune response that is difficult to induce by protein (vaccination) approaches. In order to induce a CTL response, the antigen must be presented in the context of MHC-I molecules present within the cell. Since in NAVAC the protein is expressed

15 intracellularly, such MHC-I presentation is readily achieved. Thus, nucleic acid vaccination is particularly well suited for stimulating cellular immunity, in particular, cytotoxic T cell responses to an antigen of interest.

Protein

20 The protein can be any polypeptide that has a useful therapeutic application, e.g., as a prophylactic or therapeutic vaccine. As noted above, the present invention is not limited to a particular polypeptide, however, it would be desirable to target proteins that may not intrinsically stimulate a strong cell mediated immunity response, such as polypeptides found in intracellular pathogens (for example, but not limited to

25 *M. tuberculosis*, *M. leprae*, *Chlamydia*), certain tumor-specific antigens (e.g., found on breast or colon cancer), and weakly immunogenic viral proteins (e.g., from Herpes, EBV, CMV, HPV, etc.).

It is noted that a potential drawback of recombinant proteins and subunit vaccines is the fact that glycoprotein antigens may need to be presented in a specific 30 conformation (i.e., identical to the native protein) to elicit an immunoprotective response. When purified from recombinant expression systems, it is sometimes difficult to insure that the antigens are conformationally correct. However, the

introduction of a gene encoding an antigen *in vivo* and its subsequent intracellular expression will insure that the protein product is synthesized, modified and processed very similar to the native pathogen protein. Thus, for example, the expression of a gene for a human viral glycoprotein will likely result in the correct 5 conformation of the antigen. As a consequence, the native antigenic epitopes will be presented to the immune system and can result, for example, in the generation of neutralizing antibodies specific to the native viral glycoprotein.

DNA + Protein

10 Normally a DNA vaccination elicits a predominantly Th1 helper type response. Conversely, most soluble non-particulate antigen (i.e., protein based) vaccines elicit a predominately Th2 response. Once "primed", the immune system response remains predominately Th1 or Th2, depending on the composition (i.e., nucleic acid or polypeptide) used for the initial vaccination. Quite unexpectedly the 15 combination of DNA + protein, when administered simultaneously, exhibits a more balanced Th1 + Th2 response. That is, both responses are elicited and the combination of polynucleotide/polypeptide appears to act synergistically. Moreover, the induction of CTL's is not hampered by the presence of the extracellular protein.

Based on the polynucleotide/polypeptide immune response, another advantage 20 of the instant invention is that this approach provides a method to treat latent viral infections. Several viruses (for example members of the Herpes virus group, etc.) can establish latent infections in which the virus is maintained intracellularly in an inactive or partially active form. There are few ways of treating such infections. However, by inducing a cytolytic immunity against a latent viral protein, the 25 latently infected cells can eventually be targeted and eliminated. Thus, by administering a polynucleotide/polypeptide composition, this effect should be enhanced over pure NAVAC (i.e., nucleotide only) approach since a stronger immune response, including cytolytic activity, can be established.

A related application of this approach is to the treatment of chronic pathogen 30 infections. There are numerous examples of pathogens which replicate slowly and spread directly from cell to cell. These infections are chronic, in some cases lasting years or decades. Examples of these are the slow viruses (e.g. Visna), Hepatitis B,

the Scrapie agent and HIV. Other examples of pathogens include organisms of the genus *Plasmodium*, *Mycobacterium*, *Helicobacter*, *Borrelia*, and *Toxoplasma* and well as others as noted above.

Finally, this approach may also be applicable to the treatment of malignant disease. For example, vaccination to mount a strong immune response including a T-cell mediated component to a protein expressed by the tumor cell, will potentially result in the elimination of these cells.

As used above, "simultaneous" administration refers to the same ongoing immune response. Preferably both compositions are administered at the same time (concurrent administration of both DNA + protein), however, one compound could be administered within a few week's time (preferably 1-10 days) of the other (initial) administration and still be considered as "simultaneous" since they both act during the same ongoing immune response. Normally, when a polypeptide is administered, the immune response is considered immediate in that an immune response will initiate as soon as the antigen is exposed to the immune system. In contrast, when nucleic acid is administered, peak antigen expression (*in vivo*) is observed 3-7 days after administration, and thus antigen exposure to the immune system is considered "delayed" when compared to the kinetics of protein vaccination. Regardless of this difference in kinetics, co-administration of nucleic acid and polypeptide can be considered "simultaneous" by understanding that they are both functionally present during the process of an ongoing immune response. In order to present both antigenic forms (i.e. polypeptide as such and polypeptide expressed by the administered polynucleotide), virtually simultaneously to the immune system, formulations can be conceived wherein the polypeptide is contained in such a way that its release from the formulation is delayed after the administration. This allows the expression of polypeptide from the polynucleotide to occur first, which is then subsequently complemented by the delayed released polypeptide from the formulation.

Thus another aspect of the present invention is the concurrent administration of both DNA + protein where the protein (polypeptide) is present or administered in the form of delayed-release particles intended to hide the antigen from the immune system for a short period of time. Preferably it is 1-10 days.

Regardless of the different modes or possibilities of "simultaneous" administration as described above, it is key to the invention that both antigenic substrates are present during the induction phase of an ongoing immune response. In comparison to this, the prime boost concept refers to 2 separate immune responses: (i)

5 an initial priming of the immune system with a polynucleotide followed by (ii) a secondary or boosting of the immune system with a polypeptide many weeks or months after the primary immune response has been established.

The DNA + protein complex can thus be administered as two separate events or combined (admixed) to permit one administration. Preferably, the DNA + protein 10 are admixed. Admixing can occur just prior to use, or when the two components are manufactured (and formulated), or any time in between.

The compositions and vaccines of the invention comprise DNA + protein (polypeptide) in a ratio of 1000:1 (i.e., 1 mg DNA / 1 ug protein) to 1:1 to 1:100 (w/w). Preferably it is in the range of 100:1 to 1:1. More preferably it is in the 15 range of 20:1 to 1:1. One preferred ratio is 5:1

Adjuvants

The polynucleotides, polypeptides and polynucleotide + polypeptide mixture (complex) of the present invention, when adjuvanted, are preferably adjuvanted in 20 the vaccine formulation of the invention. Vaccine preparation is generally described in *New Trends and Developments in Vaccines*, Voller *et al.* (eds.), University Park Press, Baltimore, Maryland, 1978. Fullerton describes encapsulation within liposomes, US Patent 4,235,877. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel (alum), aluminum phosphate, or 25 algammulin, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminum salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less

reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. A particularly potent adjuvant formulation involving QS21 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is a preferred formulation. In addition, the DNA could serve as an adjuvant as well

5 by encoding the gene of interest and CpG sequences. Such CpG sequences, or motifs, are known in the art.

Formulations

10 Administration of pharmaceutically acceptable salts of the polynucleotides and proteins described herein is included within the scope of the invention. Such salts may be prepared from pharmaceutically acceptable non-toxic bases including organic bases and inorganic bases. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic nontoxic bases include salts of

15 primary, secondary, and tertiary amines, basic amino acids, and the like. For a helpful discussion of pharmaceutical salts, see S. M. Berge et al., *J Pharm Sci* 66:1-19 (1977), the disclosure of which is hereby incorporated by reference.

The protein component of the formulation may be administered in the form of delayed-release particles intended to hide the antigen from the immune system for

20 several days, and to then liberate the antigen. In doing so the protein antigen is liberated once the DNA has initiated the priming of the immune response. The concept of using delayed-release formulations for achieving both priming and boosting of the immune response with protein antigens has been discussed in the literature, for example see Coombes, A. et al. (Vaccine, 14:1429-1438 (1996,)).

25 Most published attempts at achieving delayed-release formulations of protein antigens have used microparticles composed of poly(lactide-co-glycolide) in which the protein antigen is entrapped. Other formulations have been prepared with polyacrylate, latex, starch, cellulose and dextran. Another type of carrier that can be used as a delayed delivery vehicles (i.e., for delayed release formulations) are

30 supramolecular biovectors (SMBVs). SMBVs comprise a non-liquid hydrophilic core, such as a cross-linked polysaccharide or a cross-linked oligosaccharide and, optionally, an external layer comprising an amphiphilic compound, such as a

phospholipid. See, for example, US Patent 5,151,264, PCT applications WO94/20078, WO94/23701 and WO96/06638.

In a preferred embodiment of the invention the protein antigen is adsorbed to alum, and the alum particles are then coated with a bio-degradeable polymer such as 5 poly(capro-lactone) or poly(lactide-co-glycolide). Unlike published methods for encapsulating protein antigen, such formulations provide a hermetic layer of polymer around the antigen, preventing antigen liberation for a period of time ranging from a day to several weeks.

Polynucleotides and proteins for injection, a preferred route of delivery, may 10 be prepared in unit dosage form in ampoules, or in multidose containers. The polynucleotides and proteins may be present in such forms as suspensions, solutions, or emulsions in oily or preferably aqueous vehicles. Alternatively, the polynucleotide salt may be in lyophilized form for reconstitution, at the time of delivery, with a suitable vehicle, such as sterile pyrogen-free water. Both liquid as 15 well as lyophilized forms that are to be reconstituted will comprise agents, preferably buffers, in amounts necessary to suitably adjust the pH of the injected solution. For any use, particularly if the formulation is to be administered intravenously, the total concentration of solutes should be controlled to make the preparation isotonic, hypotonic, or weakly hypertonic. Nonionic materials, such as 20 sugars, are preferred for adjusting tonicity, and sucrose is particularly preferred. Any of these forms may further comprise suitable formulatory agents, such as starch or sugar, glycerol or saline. The compositions per unit dosage, whether liquid or solid, may contain from 0.1% to 99% of polynucleotide and proteinaceous material.

25 The unit dosage ampoules or multidose containers, in which the polynucleotides and polypeptides are packaged prior to use, may comprise an hermetically sealed container enclosing an amount of polynucleotide (and/or polypeptide) or solution containing a polynucleotide (and/or polypeptide) suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The 30 polynucleotide and polypeptide are packaged as a sterile formulation, and the hermetically sealed container is designed to preserve sterility of the formulation until use.

Dosage and Route of Administration of DNA/Protein Complex

The polynucleotides may be delivered to various cells of the animal body, including for example, muscle, skin, brain, lung, liver, spleen, or to the cells of the blood. Administration of the polynucleotide/protein complex is not limited to a

5 particular route or site. For example, the route could be intramuscular, intradermal, epidermal (using for example a gene gun), intra pinna, oral, vaginal, nasal, etc. Preferably the route is intramuscular, intradermal or epidermal.

Preferred tissues to target are muscle, skin and mucous membranes. Skin and mucous membranes are the physiological sites where most infectious antigens are 10 normally encountered. Skin associated lymphoid tissues contain specialized cells like keratinocytes, Langerhans' cells, and other dendritic cells which are involved in the initiation and further regulation of immune responses.

15 The dosage to be administered depends to a large extent on the condition and size of the subject being treated as well as the frequency of treatment and the route of administration. Regimens for continuing therapy, including dose and frequency may be guided by the initial response and clinical judgment. The parenteral route of injection into the interstitial space of tissues is preferred a preferred route, although other parenteral routes, such as inhalation of an aerosol formulation, may be required in specific administration, as for example to the mucous membranes for 20 the nose, throat, bronchial tissues or lungs. In addition, oral administration may be used for vaccination against infectious diseases of mucosal surfaces such as the nose, lungs and sinuses. In preferred protocols, a formulation comprising the polynucleotide/protein complex in an aqueous carrier is injected into tissue in amounts of from 10 μ l per site to about 5 ml per site. The concentration of 25 polynucleotide/protein complex in the formulation is from about 0.1 μ g/ml to about 20 mg/ml, and preferably in the range of 100-1000ug/ml.

Examples

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are meant to illustrate, but not limit the invention.

5

Example 1. Vaccine Preparation

A. Plasmid DNA

The coding sequence of the RSV-F protein (Collins et al., *Proc. Natl. Acad. Sci.*

10 81:7683-7687 (1984)) (574 amino acids) was cloned into the eukaryotic expression vector JA4304 (Lu et al., *J. Virol.* 70:3978-3991 (1996)) using cloning techniques well known by persons skilled in the art. Briefly, the RSV-F insert was cloned as a HindIII - EcoRV fragment into linearised JA4304 at the HindIII and blunt-ended BgIII restriction sites. Recombinant plasmids were analyzed by restriction digests
15 and were sequenced at the insertion sites. Plasmid DNA for injection was purified by Qiagen columns (Maxi-Prep), followed by two phenol/chloroform extractions, ether extraction, and ethanol precipitation. The plasmid DNA was resuspended into sterile water and stored at -20°C. Similarly, the coding sequence used to express the HSV gD protein (McGeoch et al., *J. Gen. Virol.* 68: 13-38 (1987)) was
20 contained in a HindIII - Bam HI - BgIII double restriction fragment, cloned into JA4304 HindIII - BgIII.

B. Protein

The coding sequence of the RSV F and G proteins (Huang et al., *Virus Res.* 2:157-25 173 (1985)) was cloned and a chimeric F/G protein was made. Very briefly, plasmid pEE14-FG containing a chimeric construct comprising of a fusion between amino acid sequences of F (1-526) and G (69-298) was received from a collaboration with A. BOLLEN (ULB/CRI, Belgium). This FG fusion protein contains a total of 755 amino acids. It starts at the N-terminal signal sequence of F
30 and lacks the C-terminal transmembrane domain (526-574) of the F glycoprotein. Then, fused to the 3' end of the coding region is the extracellular region of G glycoprotein, lacking an amino-terminal region and a carboxy terminal region. The

pEE14-FG expression plasmid was generated by the insertion of the FG coding sequence from pNIV2857 (A. Bollen, ULB/CRI, Belgium) as an Asp7181 (blunt) 5' - HindIII (blunt) 3' restriction fragment (2188 bp) into the SmaI site of pEE14 (Celltech). It will be appreciated that there are many of expression plasmids known 5 in the art that can be used.

CHO K1 cells derived were transfected with 20 ug of pEE14-FG plasmid DNA twice CsCl purified using a Ca-phosphate / glycerol transfection procedure. Cell clones were selected according to the procedure of the GS (glutamine synthetase) expression system (Crocett et al., *BioTechniques*, 8:662 (1990)) and 10 amplified in the presence of 25 micro molar methionine sulphoximine (MSX) in G MEM medium containing no glutamine and supplemented with 10% dialyzed FBS (Foetal Bovine Serum). Following transfection, 39 MSX resistant clones were screened in 24-well plates and their supernatants were tested for secretion of the FG fusion protein. All transfectants proved to be positive for F antigen expression 15 using a specific 'Sandwich' ELISA assay (i.e., rabbit polyclonal anti FG serum / FG Antigen / mAb19). Monoclonal antibody 19 recognizes a conformational F1 epitope and is neutralizing.

The 3 best FG-producer clones (n° 7, 13 and 37) were single-cell subcloned in a limiting dilution assay using 0.07 cells per well in a 96-well plates. A total of 20 59 positive subclones were obtained and the 16 best FG-producers were further characterized by western blot and ELISA. Again, the 8 best FG-subclones were further amplified and their FG expression was evaluated in presence and absence of sodium butyrate (2mM) or DMSO (1 or 2%). Six subclones were amplified and cell vials were made and stored at -80°C or in liquid N₂. Finally, the 3 best FG 25 subclones were selected. These are CHOK1 FG ° 7.18, ° 13.1, and ° 37.2.

Western blot analyses (non-reducing conditions) with monoclonal mAb19 indicated a major band of FG at about 135 kDa.

The addition of Sodium butyrate in CHO-FG cell culture medium increased the expression level of FG 3 to 12 fold depending on the subclone and cell culture 30 growth conditions. In particular, subclone CHO-FG 13.1 expressed FG protein 8-10 fold higher in the presence of butyrate.

Frozen cell culture supernatant is thawed in the cold room (4°C-7°C). Protease inhibitors (for example, 1/1000 Aprotinin, 0.5 mg/litre leupeptin, 0.5 mM Pefabloc) are added. The thawed supernatant is loaded onto a column packed with Pharmacia SP Sepharose HP™ or fast flow resin. The SP Sepharose HP™ or fast flow resin is equilibrated with 20 mM MES pH 6.5, 1% thesit (buffer D). After loading, the column is washed with buffer D until the absorbance at 280 nm returns to baseline. The column is eluted successively with buffer D containing 150 mM NaCl, than 300 mM NaCl and finally 1 M NaCl.

FG is eluted with buffer D containing 300 mM NaCl. The eluate could be, if necessary, concentrated on a Filtron OMEGA 30 or 50 kDa membrane. Eluate, whether concentrated or not, is loaded onto a Pharmacia Superdex 200™ 16/60 column equilibrated and eluted in PBS, 1% Tween 80. 1-3 mg of total protein (Lowry) are injected per run.

As noted, a truncated version of recombinant glycoprotein Herpes Simplex Virus (HSV) type 2 (rgD₂t) was also prepared. Such protein and DNA coding sequences are known in the art, see, e.g., EP 139 417B, US 5,656,457, WO 92/16231.

Recombinant Human Immunodeficiency Virus type 1 (HIV-1) envelope glycoprotein gp120 was prepared. Gp120 expression and the DNA coding sequence is known in the art as well, see, e.g., US 5,653,985, US 5,614,612, EP 290 261B.

Example 2. Immunisation of Mice

25 A. Plasmid DNA Vaccination

Plasmid DNA (encoding the RSV-F protein "DNA F") was resuspended at a concentration of 10µg/50µl of sterile PBS just prior to injection. Anaesthetized mice were injected twice with 50µl of this DNA vaccine into the tibialis anterior muscle; the booster injection was given four weeks after the first. In certain groups, the DNA vaccine was only given at the first injection.

B. Protein Vaccination

FG protein vaccine. The chimeric F/G protein ("FG") was prepared at a dose of 2 μ g/50 μ l by resuspending purified FG into 150mM NaCl, 10mM PO4 (pH 7.4). Phenoxyethanol was added as preservative at a concentration of 0.5%. The protein 5 vaccine was prepared 7 days before the primary injection, and stored at 4°C.

C. Mixed DNA/Protein Vaccination

Seven days prior to the primary injection, 2 μ g of FG per dose was mixed with 26.95 μ l H₂O and 4.25 μ l of 1.5M NaCl, 100mM PO4 (pH 7.4); in addition, 0.25 μ l 10 of phenoxyethanol was added as preservative. This preparation was stored at 4°C. Just prior to the respective injections (first or secondary), 5.65 μ l of DNA (corresponding to 10 μ g) encoding F was added to this protein preparation.

The immunisation scheme is presented below:

15

First injection	Second (booster) injection
DNA F 10 μ g	PBS
DNA F 10 μ g	DNA F 10 μ g
DNA F 10 μ g	FG 2 μ g
PBS	FG 2 μ g
FG 2 μ g	FG 2 μ g
DNA F 10 μ g +FG 2 μ g	DNA F 10 μ g +FG 2 μ g

Example 3. Serological responses upon mixed vaccine administration

Mice were immunised twice with the mixed DNA/protein vaccine composition and 20 the relevant controls, and their serological response was analysed using the following protocol.

3.a. Protocol : Enzyme-Linked ImmunoSorbent Assays (ELISA)

25 Maxisorp Nunc immunoplates were coated overnight at 4°C with 50 μ l/well of 1 μ g/ml FG antigen diluted in PBS (rows B to H of the plate) or with 50 μ l of 5

μ g/ml purified goat anti-mouse immunoglobulin antiserum (Boehringer) diluted in PBS (row A - IgG standard curve). Saturation of the plates was done for 1Hr at 37°C with 100 μ l/well of PBS BSA 1% Tween 20 0.1% NBCS 4% (saturation buffer). Then, serial, 2-fold dilutions of IgG (mouse polyclonal antiserum,

5 SIGMA) in saturation buffer (50 μ l per well) were put in rowA as standard starting at 200ng/ml; serum samples were applied in rows B to H starting at a 1/50 dilution further serially 2-fold diluted; incubation was for 1Hr30 at 37°C. Washing was done 3 times with PBS Tween 20 (0.1%). Then, biotinylated goat anti-mouse IgG antiserum (Amersham) diluted 5000x in saturation buffer was added (50 μ g/well)

10 and incubated for 1H30 at 37°C. After 3 washings as above and subsequent addition for 30 min at 37°C of streptavidin biotinylated horseradish peroxidase complex (Amersham) diluted 1000x in saturation buffer (50 μ l/well), plates were washed 5 times and incubated for 15 min at room temperature (in darkness) with 50 μ l/well of substrate solution (OPDA 0.4mg/ml and H₂O₂ 0.03% in 50 mM pH4.5

15 citrate buffer). The reaction was stopped by adding H₂SO₄ 2N (50 μ l/well). The color reaction was read on a multiscan ELISA reader at wavelength 492/620 nm. Antibody titers were calculated by the 4 parameter mathematical method using SoftMaxPro software.

The same protocol was used to determine the specific isotype distribution of the

20 antisera using mouse monoclonals (SIGMA) as standard and isotype-specific biotinylated goat anti-mouse IgG1, IgG2a and IgG2b antisera (Amersham) for the samples. The isotype distribution was expressed as the percentage of the total IgG specific immune response.

25 3.b. Results

Figure 1 (*Ig 14 post 2*) shows the serological responses in the immunised animals 2 weeks after the booster injection. The combination of the two vaccine substrates strongly enhanced the amount of RSV-FG specific antibodies compared to those

30 obtained by using the respective substrates separately ((DNAF or FG) 2x). Mixing the two vaccine compounds ((DNA + FG) 2x) was as effective as administering them in two separate injections by first priming with DNA and subsequent boosting

with the protein alone (DNAF/FG). The total IgG titer of the mixed DNA/protein vaccine was much higher than the sum of the respective titers of the separate substrates indicating synergy upon mixing of the two vaccine substrates.

Figure 2 (*IgG isotype 14 post 2*) shows the isotype distribution in the different vaccine groups. All the groups who did receive a DNA vaccine at the first injection have a strong content of IgG2a, indicative for a T helper response of type 1 (Th1), whereas the protein groups have a complete bias for the opposite, that is the IgG1 isotype typical for a Th2 type response. Surprisingly, the mixed DNA + protein group also had a high level of the IgG2a isotype. This is unexpected since the immediate presence of the protein is supposed to direct the system towards a Th2 type response and would therefore impact on the DNA vaccine, which antigen is produced *in vivo* later on. The fact that increased antibody levels were obtained (Figure 1) excludes the possibility that (i) the protein was not immunoreactive in this formulation and (ii) that the observed response was solely due to the DNA vaccine.

Example 4. Lymphoproliferative responses upon mixed vaccine administration

Induction of T helper cell activity and determination of the type of T helper cells were assessed as follows.

4.a. Protocols : *In vitro* Detection of FG-Specific Lymphoproliferation and Cytokine Production by Mice Spleen and Lymph Node Cells

25 *In vitro* stimulation

Spleens and iliac lymph nodes were taken on day 15 after second immunization. Spleens and lymph nodes of animals belonging to the same group were pooled, gently triturated in medium 1 (RPMI 1640 completed with 2 mM L glutamine + 5 10^{-2} mM 2-mercaptoethanol + 1mM sodium pyruvate + 1 x non essential MEM amino acids + 100 IU/ml penicillin + 100 μ g/ml streptomycin) (5ml/spleen cells, 1ml/lymph node cells) and centrifuged at 1200 rpm at RT for 10 minutes. Cell

pellets were then resuspended in medium 1 (3ml/spleen cells, 1ml/lymph node cells), cell suspensions were counted on a multisizer counter and adjusted to 2×10^6 cells/ml in medium 2 (medium 1 + 1% heat inactivated normal mouse serum). 100 μ l of these cell suspensions were added per well of U bottom 96 well plates.

5 For specific restimulation, 100 μ l of a serial, 2-fold dilution of purified FG (diluted in medium 2, ranging from 1.5 to 0.023 μ g/per well) was used. Controls received 100 μ l of medium only. Total stimulation was assessed by the addition of 100 μ l ConA (Boehringer) at a final concentration of 5 μ g/ml. All stimulation conditions were performed in triplicate. Plates were then incubated at 37°C in a 5% CO₂

10 incubator for respectively 48 hrs (ConA total stimulation) or 72h for FG specific restimulation. Thereafter, 100 μ l of supernatant was withdrawn out of each well and replaced by fresh medium 1 supplemented with ³H-thymidine, 1 μ Ci/well (Amersham). Plates were incubated for another 18 to 24 hr at 37°C in a 5% CO₂ incubator before being harvested. Thymidine incorporation was determined in a

15 beta counter after cell lysis on nitrocellulose filters. The results are expressed in cpm (average of triplicates) for each restimulation condition.

Cytokine detection

20 Spleen cells were processed in the same way as described here above except that they were adjusted to $5 \cdot 10^6$ cells/ml in medium 3 (medium 1 + 10 % heat inactivated FCS). 1 ml of these cell suspensions were added per well of a 24 well plate. Restimulation was done by adding purified FG antigen at 5 μ g /ml (25 μ l/well) to cells (medium was used as control). Plates were incubated at 37°C in a 5 % CO₂

25 incubator for 72 hr. Supernatants were withdrawn and tested for the presence of cytokines (IFN γ and IL-5) by Elisa. Maxisorp Nunc immunoplates were coated overnight at 4°C with 50 μ l/well of respectively 1.5 μ g/ml rat monoclonal anti-mouse INF γ (Genzyme) or 1 μ g/ml rat monoclonal anti-mouse IL5 (Pharmingen) diluted in 0.1M bicarbonate buffer (pH 9.5). Saturation of plates was done for 1H at

30 37°C with 100 μ l/well of PBS BSA 1% Tween 20 (0.1%) NBCS 4% (saturation buffer). Then, standards were applied in row A by putting serial, 2-fold dilutions of either IFN γ (Genzyme) or IL5 (Pharmingen) in saturation buffer (50 μ l per well)

starting at 2430 pg/ml or 2000pg/ml, respectively. Samples were also serially, 2-fold diluted and added to the other rows of the plate. Plates were incubated for 1H30 at 37°C. Washing was done 3 times with PBS Tween 20 0.1%. Then, biotinylated goat anti-mouse IFNg or IL5 antiserum (Genzyme and Pharmingen, 5 respectively) diluted at a concentration of 0.5 or 1.0 μ g/ml (respectively) in saturation buffer was incubated (50 μ l/well) for 1H30 at 37°C. After 3 washings as above and subsequent addition for 30 min at 37°C of AMDEX™ (Amersham) diluted 10000x in saturation buffer (50 μ l/well), plates were washed 5 times and incubated for 10 min at room temperature (in darkness) with 50 μ l/well of TMB- 10 substrate solution (Biorad). The reaction was stopped by adding 50 μ l/well of H₂SO₄ (0.4N). Color intensity was read on a multiscan ELISA reader at wavelength 450/630 nm. The results are expressed in pg/ml IFNg or IL5 referring to the corresponding calibration curves using the 4 parameters method.

15 **4.b. Results**

Figure 3 (*Lymphoproliferation*) shows the lymphoproliferation of splenocytes from the different vaccine groups upon *in vitro* restimulation with the FG antigen. Combining of the two vaccine compounds into one composition strongly increases 20 this cellular response compared to monovalent (DNA or protein) vaccines or the DNA prime + protein boost immunisation. Table 1 (*IFNg and IL5*) shows the secretion of specific cytokines, IFN gamma and IL5 respectively, from the proliferating cells. The secretion of the Th2 cytokine, IL5, is low despite the strong lymphoproliferation of the mixed vaccine group. The IFNg/IL-5 ratio (Table 25 2), being a measure of the relative ratio of Th2 to Th1 type of T helper response, is consistent with the isotype distribution of this group (see Figure 2) and further confirms the impact of the DNA in the mixed vaccine composition on the induction of a Th1 type of immune response.

30 **Example 5. Cytotoxic T cell responses upon mixed vaccine administration**

The induction of cytotoxic T cells (CTL's) was determined as follows.

Splenocytes were prepared as described in the previous section and 2.10^7 spleen cells (effector cells) were grown in a 25cm^2 flask with 10 ml of medium 3 (see example 4). These cells were stimulated for seven days (at 37°C , 7 % CO_2) by the addition of RSV infected naive spleen cells (stimulator cells) at a ratio of 10/1

5 5 (E/S). These latter stimulator cells were obtained by infecting 2.10^6 haemolyzed normal spleen cells with RSV (MOI 0.5) for 1hr30 at 37°C . In preparation of the cytotoxicity assay, EMT6 syngeneic target cells (H2d mammary adenocarcinoma cells provided by Dr B. Rouse, Knoxville) were prepared as follows. $3.x10^6$ EMT6 were collected by centrifugation, washed once with medium 1 and resuspended in

10 10 $200\mu\text{l}$ medium 1. Cells were infected with control vaccinia virus (PSC11, negative control) or a recombinant vaccinia virus expressing RSV-F at an MOI of 10 for 1 hr at 37°C (vaccinia viruses were obtained from Dr. A. Bollen, Brussels). Infected cells were then adjusted to 1 ml with medium 3 are incubated overnight at 37°C and 7% CO_2 .

15 15 Infected cells were then washed, collected by centrifugation, and resuspended in $50\mu\text{l}$ of FCS. $50\mu\text{l}$ of radiolabeled sodium chromate (DuPont) at $370\text{MBq}/\text{ml}$ was added for 1hr. Target cells were subsequently washed three times in medium 1, counted, and adjusted to a final concentration of $2.x10^4$ target cells/ml. For the cytotoxicity assay, Cr51 loaded EMT6 target cells were placed in the wells of a 96

20 20 wells microplate in the presence of different ratios of restimulated effector cells (E/T ratios of 100/1-30/1-10/1-3/1-1/1). All samples were done in duplicate at $100\mu\text{l}/\text{well}$. Microplates containing effectors and targets cells were then centrifuged for 5 minutes at 800rpm and incubated for 4 hrs at 37°C with CO_2 . Thereafter, $50\mu\text{l}$ of supernatants were transferred on a lumaplate (Packard), dried overnight and

25 25 counted. Percentage of lysis was calculated as follows : (cpm sample - cpm spontaneous release) / (cpm max (triton3% lysis)-cpm spontaneous release) x100.

Results

30 30 Figure 4 shows the presence of CTL's in the different vaccine groups. As expected, DNA vaccination induces CTL whereas non-adjuvanted FG protein has a CTL value which is the same as the background lysis towards control target cells. A

single administration of the DNA vaccine did prime for CTL induction and further boosting with either protein (alone) or DNA (alone) did not substantially increase this response. Mixing of the two vaccine compounds (DNA + protein) did not

5 impact on the induction of the CTL response by the DNA component, as CTL's could be detected. It is observed that admixing a DNA vaccine to a non-adjuvanted protein vaccine allows an extra dimension to its immune response, namely the induction of a CTL response.

10 Example 6. Enhanced humoral responses upon mixed vaccine administration are due to the presence of a specific polynucleotide encoding the polypeptide of interest

It has been shown that in certain cases plasmid DNA can exert an adjuvant effect when co-administered with a heterologous polypeptide (Sato et al., *Science* 15 273:352-354 (1996)). To exclude that the observed enhancement of the humoral response as illustrated in Example 3 was due to an aspecific adjuvant effect of the plasmid DNA, following experiment was performed. Mice were immunised as described in Example 3 with the following preparations : 10 μ g DNA-F, 10 μ g DNA-F plus 2 μ g FG (mixed antigens), 2 μ g FG, or 10 μ g pJA4304 plus 2 μ g FG. This 20 latter group resembles the mixed antigen group but with the difference that the plasmid DNA JA4304, having the same vector backbone as DNA-F, is not expressing the F polypeptide. Serological responses were analysed at 13 days after the second injection.

ANTIGEN	Average IgG (μ g/ml)	Standard Deviation
DNA-F 10 μ g	5.8	8.2
DNA-F 10 μ g + FG 2 μ g	60.8	20.5
FG 2 μ g	5.1	5.5
DNA-JA4304 + FG 2 μ g	4.4	3.9

From these results it is very clear that mixing the two specific antigen compositions (i.e. polypeptide FG and DNA-F) is strongly enhancing the humoral responses, and that a synergistic effect occurs. This enhancement is depending on the presence of a 5 plasmid encoding the antigen since a similar, non-coding plasmid is not capable of enhancing the immune response.

Example 7. A short delayed administration of a polypeptide antigen is enhancing the humoral response of the respective polynucleotide vaccine

10

Polypeptide antigens have a more rapid presentation to the immune system than polynucleotide vaccines, since the encoded polypeptide has first to be expressed after uptake of the polynucleotide by the cell. A short delayed delivery of the polypeptide antigen presents the two immunogenic compositions (virtually) 15 simultaneously to the immune system.

Mice were injected twice with the following immunogens : 30 μ g DNA-gp120, 10 μ g gp120 protein, 30 μ g DNA-gp120 followed four days later by 10 μ g gp120 protein (delayed protein delivery). Humoral responses and isotype distribution were

20 determined essentially as described in Example 3 using gp120 protein to capture the anti-gp120 specific antibodies.

Figure 5 shows the total anti-gp120 IgG titers 14 days after the second injection. IgG titers are strongly increased in the group which received the two antigen forms 25 (polynucleotide and polypeptide) where the protein administration was delayed for four days.

Figure 6 shows the isotype distribution. As can be seen, delayed protein delivery maintains considerable IgG2a titers as observed for the DNA group, and this in 30 strong contrast to the protein-only group.

Example 8. Preparation of delayed-release formulations

20 μg of the antigen gD2t was adsorbed onto 500 μg alum (aluminium hydroxide) in
5 H_2O (final volume 90 μl). A solution of poly(capro-lactone)diol (average Mn 2000,
softening temperature 50°C) in ethanol (5mg/ml: at 70°C) was added to a ratio of
10:1 (polycaprolactone:alum) and temperature allowed to equilibrate to 25°C. This
results in co-acerivation of the polycaprolactone onto the particles of alum. The
resulting coated particles were collected by centrifugation and washed with water.

10

For *in vitro* release kinetics, samples were prepared using radio-iodinated gD2t.
The gD2t was iodinated using Iodogen (Pierce) by following the manufacturer's
instructions. *In vitro* release kinetics were performed in 250 mM sodium
phosphate. This medium causes instantaneous release of gD2t from alum.

15

Polycaprolactone-coated alum was incubated in this medium at 37°C for 8 days, and
the release of antigen into the medium assayed by radioactivity measurements. For
uncoated alum, the gD2t was released into the medium within 10 minutes. For
polycaprolactone coated alum there was a slight (5-25%) release within the first 24
hours, followed by slow release over 8 days.

20

For *in vivo* studies the polycaprolactone-coated alum was mixed with DNA coding
for gD at a ratio of 2 μg protein for 10 μg DNA.

Example 9. Increased induction of humoral responses with delayed release25 formulations

In order to allow simultaneous administration of both DNA and the corresponding
protein antigen, delayed release formulations prepared as described above were
tested. Mice were injected with the following antigen compositions (ALOH-PLC
30 refers to polycaprolactone-coated alum containing gD2t):

GROUP	DAY 0	DAY 4
1	10 μ g DNA-gD	
2	10 μ g DNA-gD	2 μ g gD / AlOH
3		2 μ g gD / AlOH
4	10 μ g DNA-gD + 2 μ g gD / AlOH-PLC	
5	2 μ g gD / AlOH-PLC	

Humoral responses were determined 13 days after the first injection and are represented in the following Table:

5

GROUP	Average titer (μ g/ml)	Standard deviation	GMT
1	5.7	3.8	4.4
2	17.3	19.3	11.1
3	1	1.1	0.7
4	16	8.3	13.9
5	0.9	0.5	0.9

As can be seen from these results, combined protein and DNA immunisation leads to a stronger induction of the humoral response. The fact that titers of group 2 (protein administered 4 days after the DNA), and group 4 (delayed release

10 formulation with simultaneous injection of DNA and formulated protein) are very similar, strongly indicates that delayed release of the protein has taken place and that the concomitant presence of the protein at the time of polynucleotide directed expression strongly enhances the induction of the humoral response induced by the DNA vaccine. As can be seen, virtually simultaneous presentation of the

15 polypeptide from both vaccine compositions to the immune system can be achieved by a short delayed delivery of the polypeptide after the nucleic acid vaccination.

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

5

While the preferred embodiments of the invention are illustrated by the above, it is to be understood that the invention is not limited to the precise instructions herein disclosed and that the right to all modifications coming within the scope of the following claims is reserved.

TABLE 1:

Prime Boost RSV 14 Post 2

	IgG	IgG1	IgG2a	IgG2b	Prolif	IL5	INF _g	CTL
DNAF/PBS	1	19	51	29	1387	300	2406	16
DNAF/DNAF	2	13	77	10	1272	300	1069	22
PBS/FG	0	100	0	0	807	300	391	6
FG 2X	2	100	0	0	861	367	360	5
DNAF/FG	120	57	39	4	1837	2195	3272	15
(DNAF + FG) 2X	149	32	67	1	4190	300	1553	20
	μg/ml	%	%	%	CPM	pg/ml	% Cr51 release	

TABLE 2:

RSV 14 Post 2

	INF _g	IL5	Ratio INF _g /IL5
DNAF/PBS	2406	300	8.02
DNAF/DNAF	1069	300	3.56
PBS/FG	391	300	1.30
FG 2X	360	367	0.98
DNAF/FG	3272	2195	1.49
(DNAF + FG) 2X	1553	300	5.18
	pg/ml	pg/ml	

What is claimed is:

1. A method to enhance an immune response of nucleic acid vaccination by simultaneous administration of: (i) a polynucleotide encoding for a polypeptide of interest; and (ii) the polypeptide of interest.
- 5 2. The method of claim 1 wherein the nucleic acid is DNA.
3. The method of claim 1 wherein the nucleic acid is RNA.
4. The method of claim 2 wherein the DNA and protein are admixed.
- 10 5. The method of claim 1 wherein the polypeptide is administered 0-10 days after the polynucleotide.
6. The method of claim 5 wherein the polypeptide is administered within 3-7 days after the polynucleotide.
7. The method of claim 1 wherein the polypeptide is presented in a delayed release formulation and administered at the same time as the polynucleotide.
- 15 8. A method to enhance an immune response of polypeptide vaccination by simultaneous administration of: (i) a nucleic acid encoding for a polypeptide of interest; and (ii) the polypeptide of interest.
9. The method of claim 8 wherein the nucleic acid is DNA.
- 20 10. The method of claim 8 wherein the nucleic acid is RNA.
11. The method of claim 9 wherein the DNA and protein are admixed.
12. The method of claim 8 wherein the polypeptide is presented in a delayed release formulation and administered at the same time as the polynucleotide.
13. The method of claim 8 wherein the immune response is a Th1 response.
- 25 14. A pharmaceutical composition comprising DNA + polypeptide, wherein the DNA encodes the polypeptide of interest and wherein the ratio of DNA:Polypeptide is from 1000:1 to 1:1 (w/w).
15. The pharmaceutical composition of claim 14 wherein the polypeptide is presented in a delayed release formulation.
- 30 16. The method of claim 15 wherein the polypeptide is coated with a biodegradable polymer comprising poly(capro-lactone) or poly(lactide-co-glycolide).

17. A method to prepare a pharmaceutical formulation according to claim 14, which method comprises: purifying a polynucleotide encoding for a polypeptide of interest; purifying a polypeptide of interest; and admixing the combination thereof.
18. A method to prepare a pharmaceutical formulation according to claim 15,
- 5 which method comprises: purifying a polynucleotide encoding for a polypeptide of interest; purifying a polypeptide of interest; encapsulating the polypeptide of interest in a delayed release formulation; and admixing the combination thereof.
19. A vaccine comprising DNA + polypeptide, wherein the DNA encodes the polypeptide of interest, and wherein the ratio of DNA:Polypeptide is from 1000:1
- 10 to 1:1 (w/w).
20. The vaccine of claim 19 wherein the polypeptide is presented in a delayed release formulation.
21. The vaccine according to claim 19 comprising DNA + polypeptide and a suitable adjuvant.
- 15 22. The vaccine according to claim 20 comprising DNA + polypeptide presented in a delayed release formulation and a suitable adjuvant.
23. The use of DNA + polypeptide admixed together in the manufacture of a composition for use in enhancing the immune response of a mammal.
24. The use of DNA + polypeptide wherein the polypeptide is presented in a
- 20 delayed release formulation admixed together in the manufacture of a composition for use in enhancing the immune response of a mammal.

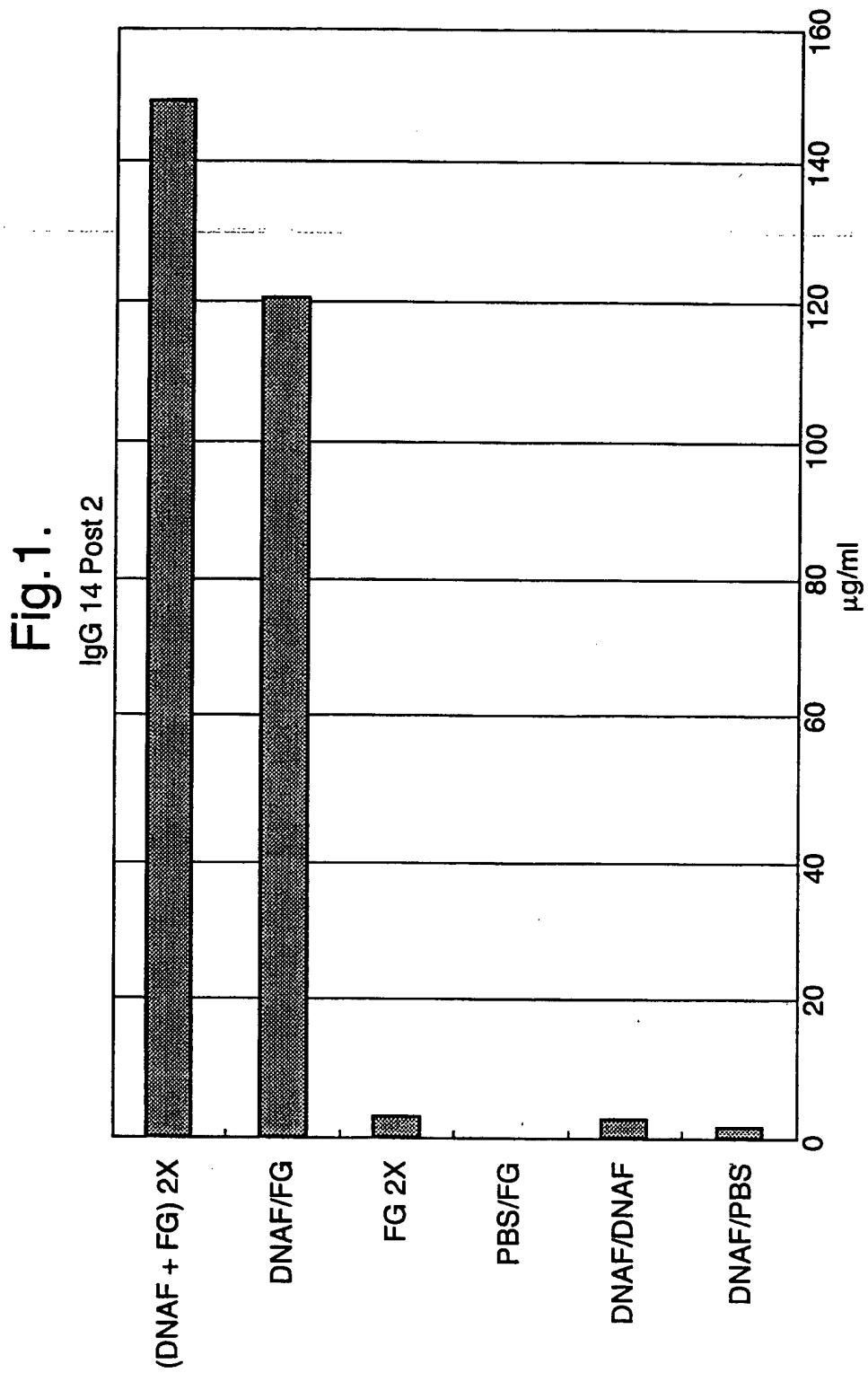


Fig. 2.
IgG Isotype 14 Post 2

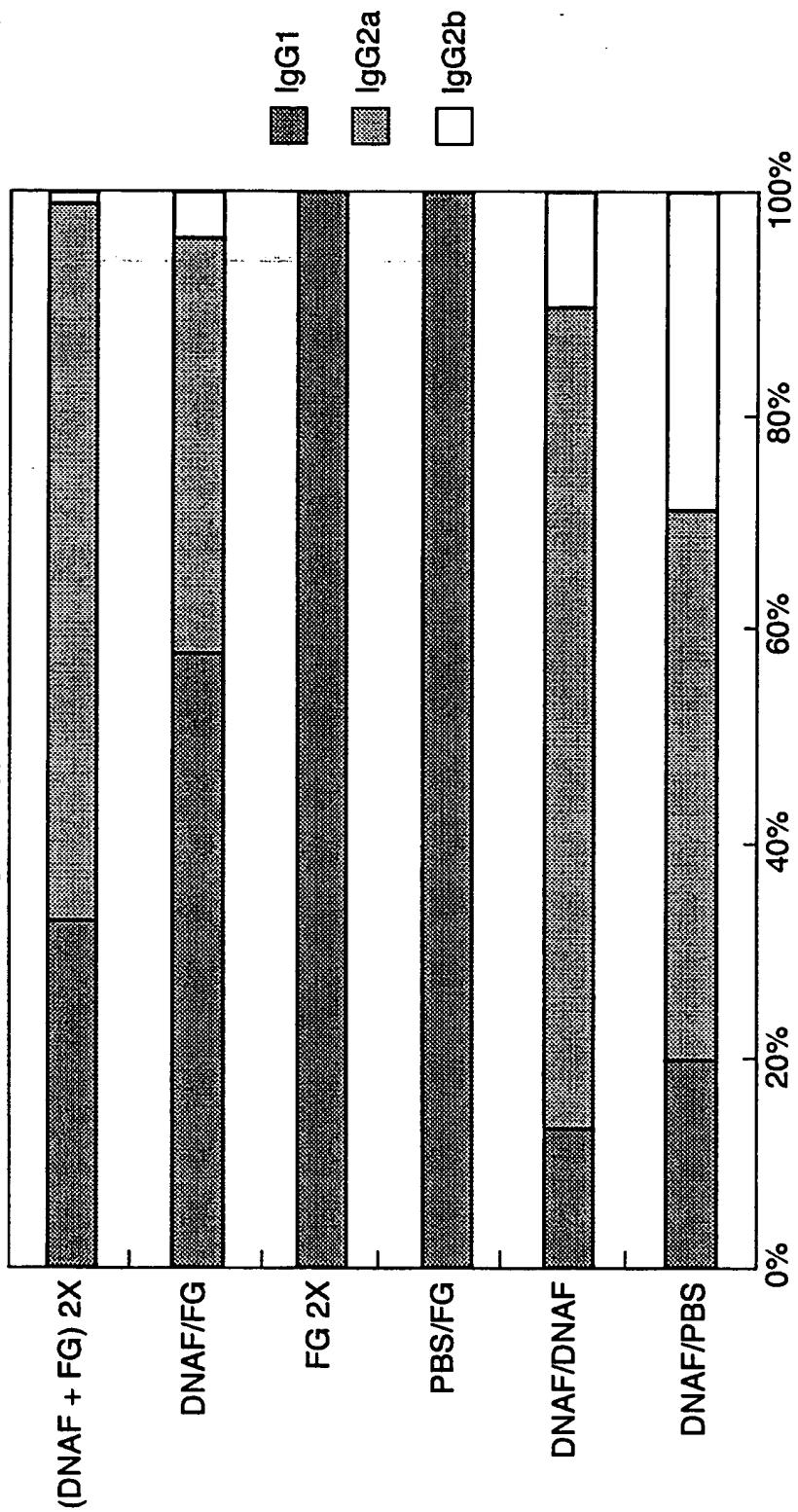


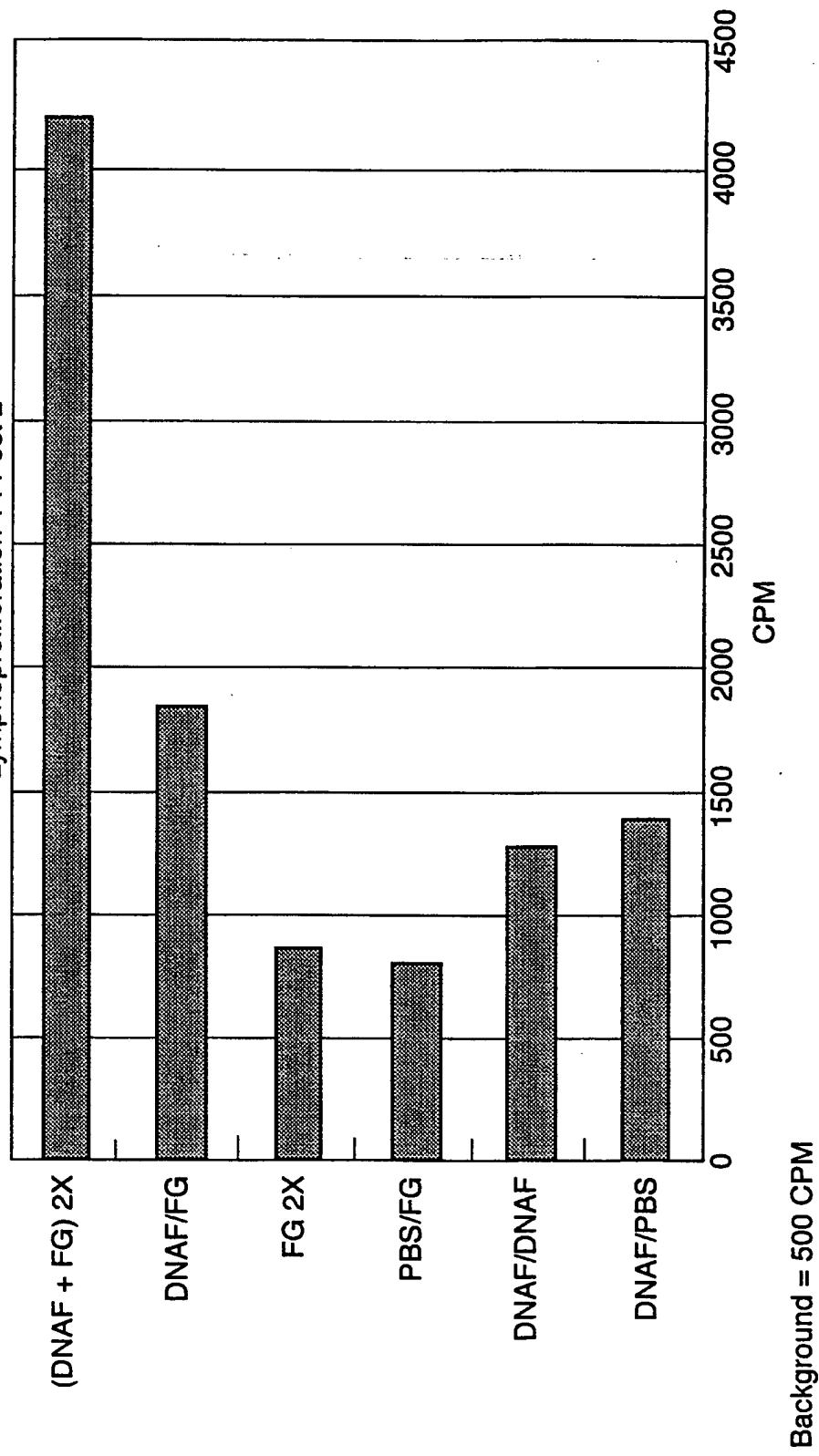
Fig.3.

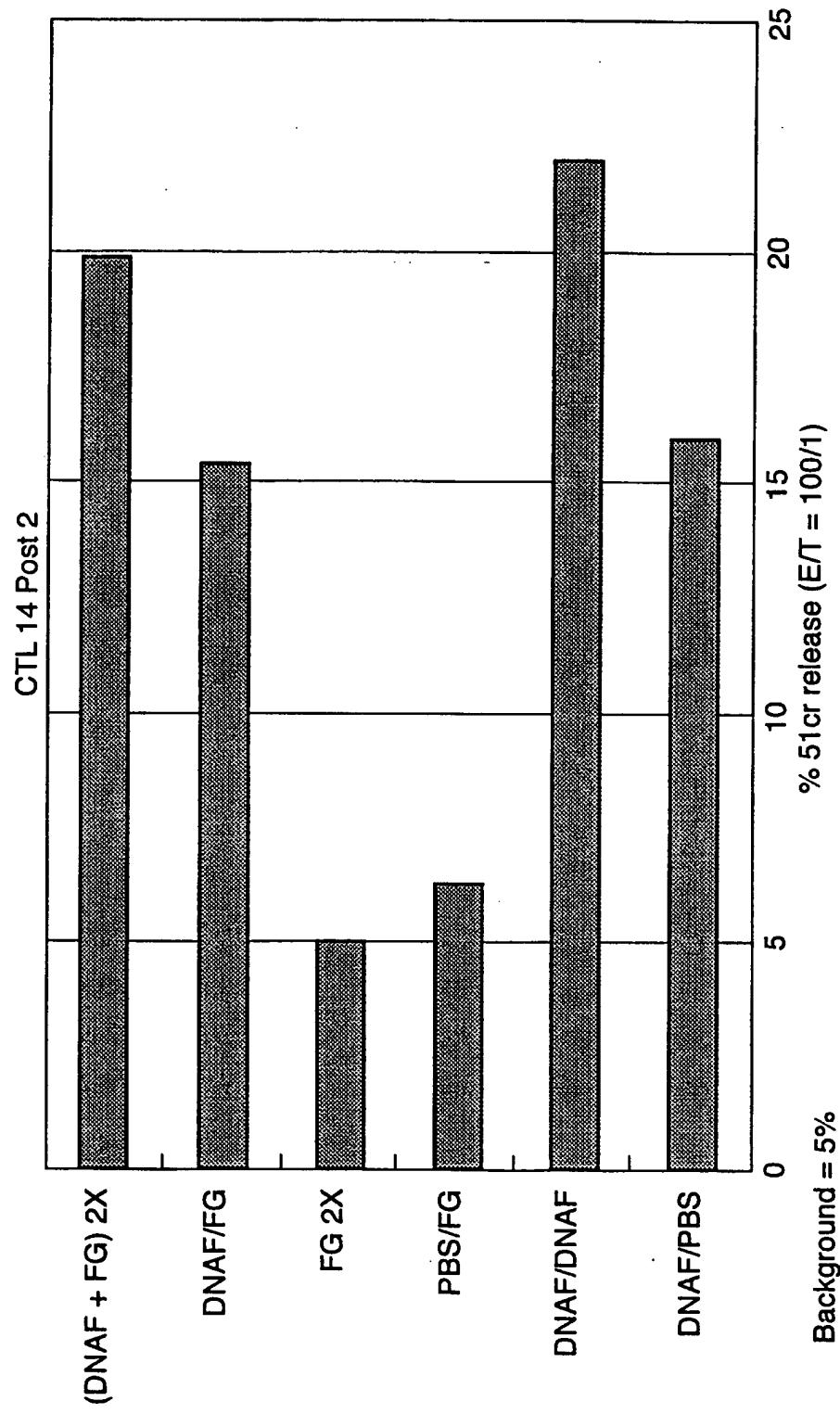
Fig. 4.

Fig.5.

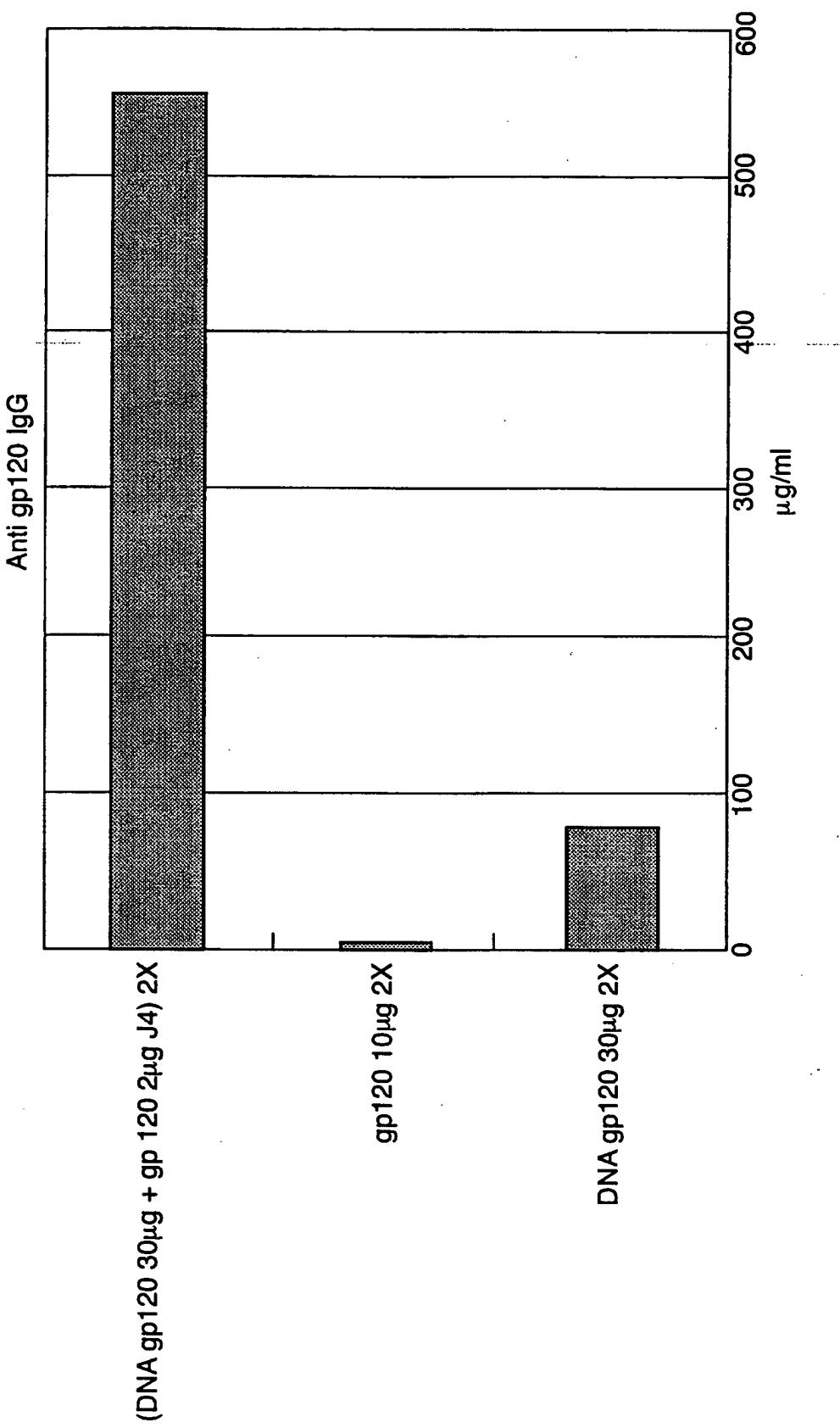
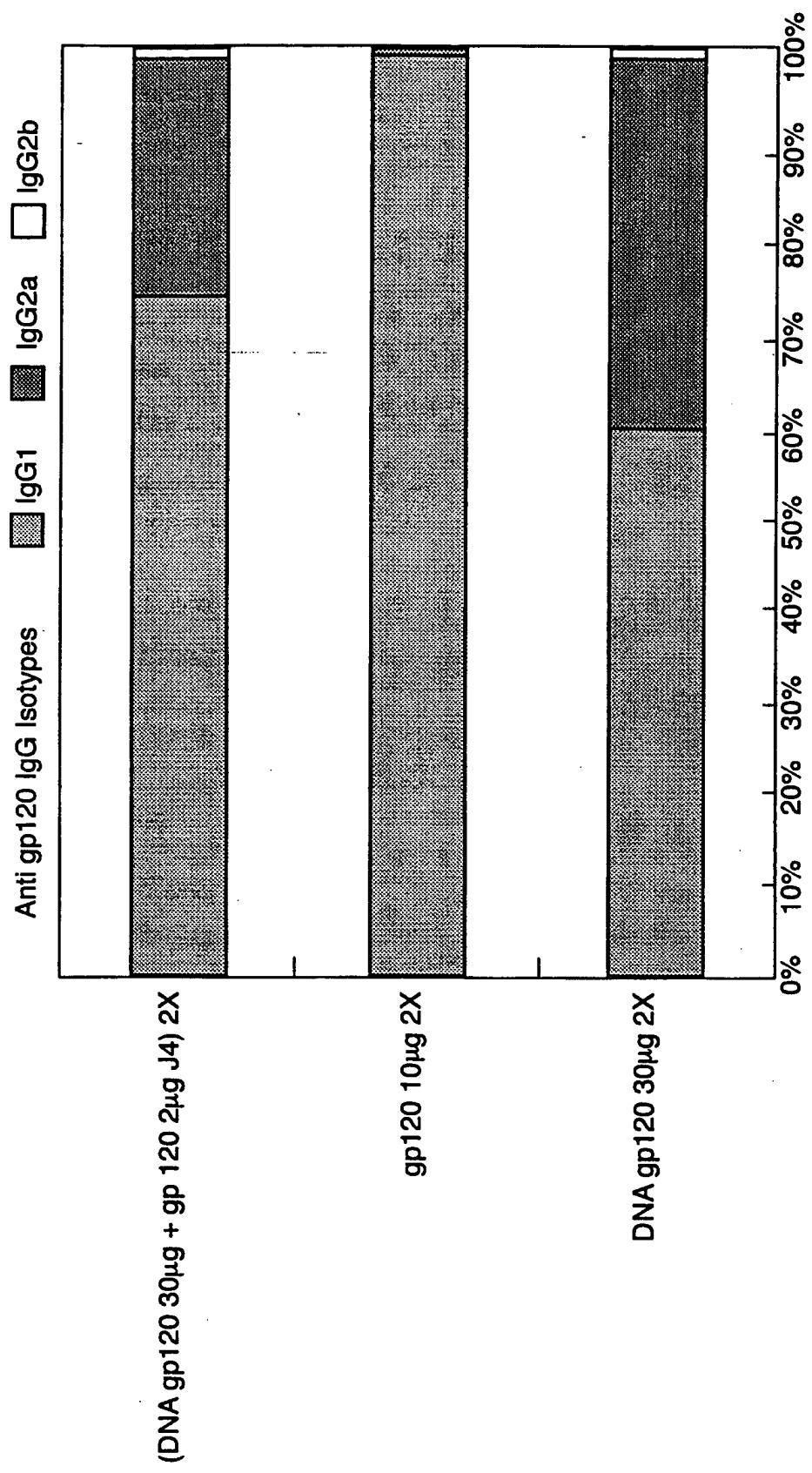


Fig. 6.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/08152

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K39/00 A61K31/70 A61K39/39 A61K9/00 // (A61K39/00, 31:70)				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	LETVIN N L ET AL: "Potent, protective anti- HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 AUG 19) 94 (17) 9378-83. JOURNAL CODE: PV3. ISSN: 0027-8424., XP002097492 United States see page 9378, left-hand column, paragraph 2 see page 9378, right-hand column, paragraph 2-3 see page 9380, left-hand column - right-hand column, paragraph 2 see page 9382, right-hand column, paragraph 1-3 --- -/-	1-6, 8-11,13, 14,17, 19,23		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed				
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents such combination being obvious to a person skilled in the art "Z" document member of the same patent family				
Date of the actual completion of the international search 21 May 1999		Date of mailing of the international search report 01/06/1999		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Covone, M		

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/08152

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 28818 A (THEREXSYS LTD) 14 August 1997 see page 2, line 18-22 see page 3, line 33 - page 4, line 32 see page 12, line 10 - page 13, line 14 see page 52, line 1 - page 53, line 8 see page 64, line 25 - page 66, line 14 see claims 1-4 ---	1-14, 17, 19-24
X	OKUDA K ET AL: "DNA vaccination followed by macromolecular multicomponent peptide vaccination against HIV-1 induces strong antigen-specific immunity" VACCINE, vol. 15, no. 10, 1 July 1997, page 1049-1056 XP004085962 see page 1050, left-hand column, paragraph 1-2 see page 1054, right-hand column, line 40-43 ---	1-6, 8-11, 13, 14, 17, 19, 23
A	WO 96 40066 A (UNIV TORONTO ;SAMUEL JOHN (CA); KWON GLEN S (CA)) 19 December 1996 see page 4, line 29 - page 6, line 29 see examples 6,12 ---	7, 12, 15, 16, 18, 20-22, 24
A	DUMMY: "Pharmaceutical materials for treatment of papilloma virus -associated lesions; interleukin-12 expression using a recombinant herpes virus with vaccinia virus antigen, for application as a recombinant vaccine" VACCINE, vol. 15, no. 3, 1 February 1997, page 340 XP004094421 see the whole document ---	3, 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 98/08152

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 1_13
because they relate to subject matter not required to be searched by this Authority, namely:
See FURTHER INFORMATION SHEET PCT/ISA/210

2. Claims Nos.: 16
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claim 16 reads "the method of claim 15". However claim 15 is directed to a pharmaceutical composition. This is obscure. Therefor claim 16 has been searched as if it was directed to the pharmaceutical composition of claim 15 wherein the polypeptide is as defined in claim 16.

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 1-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Claims Nos.: 1-13

Rule 39.1(iv) PCT = Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/08152

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9728818	A 14-08-1997	AU 1800597	A 28-08-1997	CA 2244110 A 14-08-1997
		EP 0880360	A 02-12-1998	
WO 9640066	A 19-12-1996	AU 6330896	A 30-12-1996	

**HPS Trailer Page
for**

EAST

**UserID: AWehbe_Job_1_of_1
Printer: cm1_12e14_gbejptr**

Summary

Document	Pages	Printed	Missed	Copies
WO009930733	42	42	0	1
Total (1)	42	42	0	-